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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,235,420**, on June 17, 1998, by **PAOLO RENZI**, for "Antisense Oligonucleotides for Treating or Preventing Atopic Diseases and Neoplastic Cell Proliferation".

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ABSTRACT OF THE INVENTION

The present invention relates to the use of antisense oligonucleotides directed against specific nucleic acid sequences coding for receptors, alone or in combination, in order to inhibit the inflammatory reaction that is present in asthma, atopy or hypereosinophilia and to inhibit neoplastic cell proliferation. The antisense oligonucleotides of the present invention are used for treating and/or preventing asthma, allergy, hypereosinophilia, general inflammation or cancer. The oligonucleotides of the present invention are more specifically directed against nucleic acid sequences coding for a CCR3 receptor, a common subunit of IL-4 and IL-13 receptors, or a common subunit of IL-3, IL-5 and GM-CSF receptors.

ANTISENSE OLIGONUCLEOTIDES FOR TREATING OR PREVENTING ATOPIC DISEASES AND NEOPLASTIC CELL PROLIFERATION

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the use of antisense oligonucleotides directed against specific cellular receptors, alone or in combination, in order to inhibit the inflammatory reaction that is present in asthma, hypereosinophilia or atopic diseases and to inhibit neoplastic cell proliferation.

(b) Description of Prior Art

Antisense oligonucleotides are a new class of pharmaceuticals. In general, antisense refers to the use of small, synthetic oligonucleotides, with the same constituents as that found in our own DNA and which resemble single stranded DNA. The antisense oligonucleotides are designed as a mirror sequence of a part of a gene they are targeting in order to be able to adhere to this sequence and inhibit gene expression. Gene expression is inhibited through hybridization of sense oligonucleotide to a specific messenger RNA (mRNA) sense target according to the Watson-Crick base pairing in which adenosine and thymidine or guanosine and cytidine interact through hydrogen bonding. These simple base-pairing rules govern the interaction between the antisense oligonucleotides and the cellular RNA, which allow to design an antisense oligonucleotide. A major advantage of this new strategy is the specificity of action with the potential for less side effects and toxicity. This therapeutic strategy could potentially be applied to any disease where an overexpression of one or several genes are believed to cause the presence or persistence of the disease. As a result, there have been numerous

studies of antisense oligonucleotides as therapeutic agents for cancer and viral diseases.

Few studies have been performed in order to assess whether antisense oligonucleotides could inhibit receptor expression on cell surfaces for inflammatory mediators.

Antisense oligonucleotides can be used to inhibit interleukin(IL)-6 receptor expression and thus the effects of the acute inflammatory mediator interleukin-6 on cells. No studies have been conducted to assess whether antisense oligonucleotides can be employed to inhibit receptors on cells that are involved in asthmatic inflammation or on cancerous cells.

Asthma is a disease that affects 5 to 10% of the population which has doubled in prevalence in the last 25 years. This increase has been noted especially in infants after a viral infection of the airways (bronchiolitis), in children and in occupational induced asthma. The exact cause of asthma is not yet known. However, it is believed that agents such as viruses are involved in the perpetuation of the abnormal inflammation that is found in the airways of patients with asthma and thus the persistence of the disease.

For this reason the current recommendations for first line therapy of asthma is a potent anti-inflammatory medication such as corticosteroids and antileukotrienes. Although this therapy is effective in many patients, some patients are resistant to corticosteroids. This medication is also a potent immunosuppressive with long term side effects and has not been shown to be effective in the prevention of allergy or asthma.

Antileukotrienes have some effect in allergy and asthma but are not as effective as corticosteroids.

Several inflammatory mediators play a role in the appearance and perpetuation of inflammation in the airways of patients with asthma. Some mediators attract the inflammatory cells into the airways either through chemotaxis of eosinophils (the chemokines: rantes, eotaxin 1,2, MCP-3,4 that act mostly in asthmatic inflammation through a receptor called CCR3) or through endothelial cell activation (IL-4,13). Other mediators cause the priming and increased survival of inflammatory cells in the airways (IL-3,5, GM-CSF, IL-4). These mediators thus consist of either specific chemokines for eosinophils or of cytokines of the T helper lymphocyte type 2 phenotype (Th2: IL-3,4,5,13 and GM-CSF).

An improvement in asthma has been shown when there is a decrease in these inflammatory mediators in the airways.

Allergy is a disease that is extremely prevalent, for example atopic rhinitis affects around 30% of the population. Allergy is characterized by abnormal IgE production and inflammation to an allergen. In the presence of IgE and allergen, effector cells such as the mast cells degranulate and release inflammatory mediators leading to the recruitment of the same inflammatory cells that are found in asthma. In atopic rhinitis, nasal polyposis and chronic sinusitis one finds the same excess in inflammatory mediators as those present in asthma. IL-4 and IL-13 are necessary for the production of IgE and the induction of the cells with a Th2 phenotype.

Cancer is the second cause of death in humans and is characterized by abnormal proliferation of immortalized cells. One of the mechanisms that is

involved in the persistence and increase in these cells is by the release of growth factors that act through receptors and lead to cellular proliferation. Amongst these growth factors, GM-CSF has been shown to be an important growth factor for several tumor cells. The inhibition of proliferation of cancerous cells by blocking the receptors for growth factors could be important in the therapy of certain cancers.

It would be desirable to be provided with the use of antisense oligonucleotides directed against at least one specific common receptor for either Th2 cytokines or receptor for mediators that attract cells that respond to Th2 cytokines, in order to inhibit the inflammatory reaction that is present in asthma or atopy and to inhibit neoplastic cell proliferation.

It would also be highly desirable to be provided with antisense oligonucleotides directed against a nucleic acid sequence coding for receptors so that by inhibiting these receptors these oligonucleotides could be employed in the therapy and/or prevention of asthma, allergy, general inflammation and cancer.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide the use of antisense oligonucleotides directed against at least one common subunit of a cellular receptor, such as the common subunit for IL-3, IL-5, and GM-CSF, or the common subunit for the IL-4 and IL-13 or the CCR3, in order to inhibit the inflammatory reaction that is present in asthma or atopy and to inhibit neoplastic cell proliferation.

Another aim of the present invention is to provide antisense oligonucleotides directed against a nucleic acid sequence coding for the common subunit of

the IL-4 and IL-13 receptors so that by inhibiting these receptors these oligonucleotides could be employed in the treatment and/or prevention of asthma, allergy, general inflammation or cancer.

Another aim of the present invention is to provide antisense oligonucleotides directed against a nucleic acid sequence coding for the common beta subunit of the IL-3, IL-5 and GM-CSF receptors so that by inhibiting these receptors they may be employed in the treatment and prevention of asthma, allergy, hypereosinophilia, general inflammation or cancer.

Another aim of the present invention is to provide antisense oligonucleotides directed against a nucleic acid sequence coding for the CCR3 receptor for chemokines so that by inhibiting these receptors they may be employed in the treatment and prevention of asthma, allergy, general inflammation or cancer.

Another aim of the present invention is to provide a therapeutically effective composition comprising at least two antisense oligonucleotides directed against nucleic acid sequences coding for the common subunit of IL-4 and IL-13 or the common beta subunit of IL-3, IL-5, and GM-CSF, or the CCR3 receptors for a more potent effect in the treatment and/or prevention of asthma, allergy, general inflammation or cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates increased IL-4 production in response to the house dust-mite antigen is increased in subjects with early wheezing;

Figs. 2A and 2B illustrate cytokine production during bronchiolitis as a predictor of the severity of wheezing;

Figs. 3A and 3B illustrate the relationship between IFN production in response to IL-2 by blood mononuclear cells and the development of asthma 2 years after bronchiolitis in infants;

Figs. 4A and 4B illustrate the correlation between interferon gamma production in response to IL-2 at the time of bronchiolitis in infants and Vmax FRC (4A) or PC40 histamine (4B);

Figs. 5A to 5C illustrate the distribution of an FITC labeled antisense phosphorothioate oligonucleotide 8 hours after being nebulized or breathed into the lungs of a rat;

Figs. 6A and 6B illustrate inflammatory cells (Fig. 6A) and an FITC-labeled antisense phosphorothioate oligonucleotide which has found its way into the inflammatory cells (green fluorescence)(Fig. 6B) retrieved from lung lavage of rats 24 hours after administration;

Figs. 7A and 7B illustrate gels showing the antisense phosphorothioate oligonucleotides still intact when retrieved from the bronchoalveolar lavage (BAL)(Figs. 6A and 6B) and from lungs (Fig. 6B) of rats 24 hours after administration when compared to a control antisense oligonucleotide;

Fig. 8 illustrates the antisense phosphorothioate oligonucleotides OD1, OD2 and OD3 inhibiting IL-4 and IL-13 receptor expression in RAJI cells as detected by Flow cytometry;

Figs. 9A and 9B illustrate the antisense phosphorothioate oligonucleotides OD1, OD2 and OD3 in accordance with one embodiment of the invention, inhibiting protein expression of the IL-4 receptor in RAJI cells as detected by immunoprecipitation and Western;

Figs. 10A to 10F illustrate the dose response of the antisense oligonucleotide OD2 at inhibiting protein expression of the IL-4 and IL-13 receptor in RAJI cells as detected by immunochemistry;

Figs. 11A and 11B illustrate the antisense phosphorothioate oligonucleotide 107A inhibiting mRNA expression (by semi-quantitative RT-PCR) of the common beta subunit of the IL-3, IL-5 and GM-CSF receptor in TF1 (Fig. 11A) and U937 (Fig. 11B) cells;

Fig. 12 illustrates the antisense phosphorothioate oligonucleotide 107A inhibiting protein expression of the common beta subunit of the IL-3, IL-5 and GM-CSF receptors in TF1 cells as detected by immunoprecipitation and Western;

Fig. 13 illustrates the antisense phosphorothioate oligonucleotide 107A inhibiting as a dose response TF1 cell proliferation;

Fig. 14 illustrates the antisense phosphorothioate oligonucleotide 107A inhibiting TF1 cell growth;

Figs. 15A to 15H illustrate the expression and cellular distribution of eotaxin mRNA (Figs. 15A to 15D) and protein (Figs. 15E to 15H) in airways (Figs. 15A, 15B, 15E) and BAL cells (Figs. 15C, 15D, 15F to 15H) of allergic asthmatic patients (Figs. 15A, 15C, 15E, 15F, 15G) and normal controls (Figs. 15B, 15D, 15H);

Figs. 16A to 16H illustrates expression and cellular distribution of MCP-4 mRNA (Figs. 16A, 16B, 16E, 16F) and protein (Figs. 16C, 16D, 16G, 16H) in airways (Figs. 16A to 16D) and BAL cells (Figs. 16E to 16H) of allergic asthmatic patients and normal controls;

Fig. 17 illustrates the effect of preincubation with IL-5 on the chemotaxis induced by eotaxin; and

Figs. 18A to 18I illustrate the effect of preincubation with IL-5 overnight on chemokine production.

DETAILED DESCRIPTION OF THE INVENTION

Bronchiolitis is a viral infection of the airways of infants that predisposes to the development of asthma. This condition was studied since it is the earliest one can get in humans prior to the development of asthma, atopy, and allergic inflammation. As is shown hereinafter, an imbalance in the Th1 to Th2 cytokine ratio, favoring Th2 cytokines, is present prior to developing asthma. In one embodiment, the present invention aimed at restoring this imbalance and thus at preventing or treating asthma and allergies.

Results obtained in lymphocytes isolated from infant blood suffering from bronchiolitis have suggested and confirmed that an imbalance exists between Th1 and Th2 cytokine production prior to the development of early wheezing. Indeed, Fig. 1 shows that lymphocytes from infants who wheeze after bronchiolitis have an increased production of IL-4 (a Th2 cytokine) after exposure to the house dust-mite antigen. In Fig. 1, lymphocytes were isolated from the blood of infants 5 months after bronchiolitis and cultured in the presence of the house dust-mite antigen. IL-4 was measured in the supernatant collected 3 days after culture. Results are presented for the subjects who wheezed for at least one of the last 90 days and those who did not wheeze at all within the first 5 months after bronchiolitis. Asterisk indicates subject who had an IL-4 level of 535 pg/ml. In addition, in infants that wheezed the most in the first five months after bronchiolitis a lower

interferon gamma (IFN, a Th1 cytokine) production and a higher IL-4 production was found.

The condition of these infants were monitored for 2 years, pursuant to which it was determined whether they had no asthma, possible asthma or probable asthma by the Delphi consensus. The smoking history and the presence of atopy or asthma in parents or siblings were recorded and blood mononuclear cell IFN and IL-4 production in response to IL-2 were assessed in 32 infants hospitalized for bronchiolitis and in a sub-group (n=19) in which pulmonary function tests were performed 4.9 months later.

In Figs. 2A and 2B, lymphocytes were isolated from subjects during bronchiolitis and cultured in the presence of IL-2 for 3 days. The supernatant was collected and the cytokines measured by ELISA. Results are presented for the subjects who wheezed more than 20 days (more wheezing, n=9) and those who wheezed for fewer than 20 days (less wheezing, n=6).

Infants with possible and probable asthma had lower IFN production at the time of, and 4.9 months after bronchiolitis when compared to those who had no asthma ($p<0.05$, Figs. 3A and 3B).

In Figs. 3A and 3B, mononuclear cells obtained at the time of bronchiolitis (3A, n=32) or 4.9 months later (3B, n=19) were partially depleted of monocytes and cultured with IL-2 for 3 days. The supernatant was retrieved and IFN production was measured by ELISA. Results are presented for patients evaluated 2 years after bronchiolitis as having no asthma (no), possible asthma (possible) and probable asthma (probable). For results identified by "*", a probability of $p<0.05$ was found using the Kruskall-Wallis test and Mann-Whitney U test possible and probable versus no asthma. For results identified by "**", a probability of $p=0.08$ was

found using the Kruskall-Wallis. For results identified by "++", a probability of $p<0.05$ was found using the Mann-Whitney U test possible and probable asthma versus no asthma.

IL-4 production did not differ between groups. Significant positive correlations were found between IFN production at the time of bronchiolitis and markers of abnormal airway function (Vmax of functional residual capacity (FRC), Fig. 4A)) or of increased airway responsiveness (PC40 histamine, Fig. 4B)), 4.9 months after bronchiolitis.

In Figs. 4A and 4B, cytokine production was measured at the time of bronchiolitis and pulmonary function was measured 4.9 months later. Pulmonary function was evaluated with methods recommended by the American Thoracic Society. Maximal expiratory flow at functional residual capacity (Vmax FRC) was assessed by the rapid thoracoabdominal compression technique (RTC) using the following procedure. Patients previously sedated with chloral hydrate 100 mg/Kg body weight (maximal dose 1000 mg) were placed supine with the neck slightly extended in an inflatable jacket covering the abdomen and thorax and connected to a pressure reservoir. Starting from a pressure of 30 cm H_2O and using increments of 5 cm H_2O, measurements of expiratory flow at FRC were obtained until Vmax FRC was achieved. Flows were measured with a soft cushion mask connected to a Fleisch no. 1 pneumotachograph and integrated. Three additional technically correct maneuvers were performed at this pressure from which the highest value was chosen to represent baseline Vmax FRC. All subsequent Vmax FRC maneuvers were carried out using the same procedure.

Bronchial reactivity to histamine was assessed by using a Hudson updraft #2 nebuliser driven at 8

liters/min. to administer doubling concentrations of histamine starting at 0.0625 mg/ml to a maximum of 8.0 mg/ml for 1 minute at 5 minute intervals. Vmax FRC was determined after each nebulization. The challenge test ended when a decrease in Vmax FRC of at least 40% from baseline value had been reached, or the maximum concentration of histamine had been given. Heart rate and oxygen saturation were continuously monitored throughout the study with an Ohmeda BIOX 3740 pulse oximeter.

A defect in IFN production is a primary contributor to the development of asthma in infants. Interestingly, this defect is present in adults with asthma and in newborns before they develop atopy. There thus is an imbalance in the relative production of Th2 (IL-4, IL-13, IL-5, etc.) vs. Th1 (IFN) cytokines that is present even before one develops asthma or allergy, the ratio of Th2 over Th1 cytokines is increased prior to the development of and during these diseases.

In order to treat or prevent the development of allergy, asthma or neoplastic cell proliferation that is dependent on an abnormal increase in the production or the effects of Th2 cytokines, it was thus found desirable to decrease the effects of the Th2 cytokines.

Accordingly, there is provided hereinafter evidence that antisense oligonucleotides according to one embodiment of the present invention, which are breathed into the lungs, are deposited therein, and enter cells where they are active and remain in a non-degraded and thus potent state for at least 24 hours (See Figs. 5 and 6 and Example I).

Antisense oligonucleotides according to a preferred embodiment of the present invention are directed against the common subunit of the IL-4 and IL-

13 receptors. These antisense oligonucleotides are effective at inhibiting the functional subunit of these receptors, as illustrated in Example II.

Antisense oligonucleotides in accordance with another embodiment of the present invention are directed against the common beta subunit of the IL-3,5 and GM-CSF receptors. These antisense oligonucleotides are effective at inhibiting these receptors and thus at preventing the proliferation or function of cancerous or inflammatory cells that depend on these growth factors for survival (See Example III).

Antisense oligonucleotides in accordance with another embodiment of the present invention are directed against the CCR3 receptor of chemokines. These antisense oligonucleotides are effective at inhibiting this receptor and thus at preventing the influx, survival and proliferation or function of inflammatory cells and cancerous cells or infectious organisms that depend on this receptor (See Example IV).

The present invention will be more readily understood by referring to the following examples which are given to illustrate the following invention rather than to limit its scope.

EXAMPLE I

Effective administration of antisense oligonucleotides

In order for any therapy to be effective, the administered substance must first find it's way into the lungs and to the cells where it is to have its effects and second, to remain intact without having any side effects. Antisense oligonucleotides breathed into the lungs, are deposited in the lungs and airways to enter the cells where they have their effects and remain in a non-degraded state for at least 24 hours without affecting lung physiology. One microgram

(1 µg) of antisense phosphorothioate oligonucleotide of the present invention that had previously been tagged with FITC was administered by nebulization into the lungs of rats. Rats were anesthetized with urethane (1 g/kg, i.p.). A heating pad was used to maintain body temperature constant during the experiment and rectal temperature was monitored continuously with an electronic thermometer. After blind orotracheal intubation with 6 cm of PE-240 polyethylene catheter, pulmonary resistance was measured during spontaneous tidal breathing with the animals in the supine position. Flow was measured by placing the tip of the tracheal tube inside a small Plexiglas® box (265 ml in volume). A Fleisch no. 0 pneumotachograph coupled to a piezoresistive differential pressure transducer (Micro-Switch 163PC0ID36, Honeywell, Scarborough Ont. Canada) was attached to the other end of the box to measure airflow. Transpulmonary pressure (Ptp) was measured using a water-filled catheter placed in the lower third of the esophagus connected to one port of a differential pressure transducer (Transpac II, Abbott, Illinois), the other port being connected to the Plexiglas box. The esophageal catheter consisted of a polyethylene tube (PE-240, 10 cm long) with a terminal tip (6 cm) of a smaller bore tube (PE-160).

The pressure and flow signals were amplified, passed through eight-pole Bessel filters (9 model 902LPF, Frequency Devices, Haverhill, MA) with their cut off frequencies set at 100 Hz. The data were stored on a computer. Lung resistance was calculated by multiple linear regression by fitting the equation of motion as performed with commercial software (RHT Infodat Inc. Montreal, PQ).

After instrumentation, an aerosol of saline containing 1 µg of the tagged phosphorothioate

oligonucleotide was administered for five minutes. This was generated using a Hudson nebulizer with an output of 0.18 ml/min. connected to one side port of the box. The box was flushed with a stream of fresh air between measurements in order to prevent the accumulation of CO_2. Lung resistance was measured 5, 10, 15, 20 and 30 minutes after challenge and subsequently every 15 minutes for a total time of 8 hours. Lung resistance did not change over this time period. The rats were then killed by exsanguination and the lungs retrieved to determine whether the oligonucleotide was still present. The lungs were fixed in paraformaldehyde and an anti-FITC antibody tagged with alkaline phosphatase was used to determine the site of the oligonucleotide, the tissue samples were revealed with fast red and the nucleus of the cells counterstained with a Hoechst counterstain. It is to be noted in Fig. 5A that the oligonucleotides (in red) are present diffusely in all cell types. The oligonucleotides have penetrated the cytoplasm of the cells (5B) and are also found in an inflammatory cell (macrophage, in the middle of 5C).

In other experiments, the rats were anesthetized with pentothal and awakened after antisense nebulization. Bronchoalveolar lung lavage (BAL) was performed 24 hours later after general anesthesia by administration of 5 ml of saline and gentle aspiration. The BAL was centrifuged at 400xg for 10 minutes, the supernatant frozen and the cells centrifuged onto slides for analysis. It is to be noted in Fig. 6A that macrophages are the predominant cell type. The FITC-labeled oligonucleotide (green fluorescence) in Fig. 6B is present in the cytoplasm of the cells. The FITC-labeled oligonucleotide was either extracted from the lavage or the lungs of the rats 24

hours after antigen challenge. It is to be noted in Fig. 7A that the phosphorothioate oligonuleotide is still intact when extracted from the lung 24 hours after administration, lane 3 compared to 2 µl of the purified oligonucleotide in lane 2 and the standard (lane 1). It is to be noted in Fig. 7B that the antisense oligonucleotide is also intact 24 hours after administration in the BAL (lane 1), lung (lane 2) when compared to it's own control (lane 3) or another oligonucleotide that is tagged with FITC (eotaxin, lane 4).

As can be shown from Figs. 5 to 7, the antisense oligonucleotides of the present invention are breathed into the lungs, to penetrate the cells, remaining intact for more than 24 hours.

EXAMPLE II
Antisense oligonucleotides inhibiting the common subunit of the IL-4 and IL-13 receptors

Interleukin-4 is involved in IgE production, the development and persistence of asthma and atopy. Although therapies directed against the effects of IL-4 may be effective in the prevention of asthma, allergy or neoplastic cell proliferation (that depends on this mediator), it has recently been shown that another Th2 cytokine (IL-13) has the same effects as IL-4. Interestingly IL-4 and IL-13 share a common subunit which is necessary for signal transduction of the message to occur.

Experiments were performed to assess whether antisense oligonucleotides directed against the common subunit of the IL-4 and IL-13 receptor could inhibit the expression of this receptor. RAJI cells express high levels of IL-4 and IL-13 receptors. These cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin

and l-glutamine at 37°C in 5% CO_2. For 12 hours the cells were either cultured in medium alone or medium with sense or antisense oligonucleotides to the common subunit of IL-4/IL-13. The cells were retrieved, washed 3 times and then stained with an anti-human IL-4 receptor antibody (R and D systems, catalog number MAB230), which has been shown to block the human cell surface receptor-mediated bioactivities caused by IL-4 or IL-13. It is to be noted in Fig. 8 that the antisense oligonucleotide OD1: 5'-agaccttcat gttcccagag-3' (SEQ ID NO:1), OD2: 5'-gttcccagag cttgccacct-3' (SEQ ID NO:2) or OD3: 5'-cctgcaagac cttcatgtt-3' (SEQ ID NO:3) inhibits the expression of the bioactive form of the IL-4 receptor. The first line shows the absence of fluorescence in cells that were either unstained (left) or exposed to a non-specific monoclonal antibody (right). The second line shows that RAJI cells express the IL-4 receptor (92%) and that the fluorescence intensity is very high (many receptors). On the right, the RAJI cells were incubated for 12 hours with 10 µMol of the antisense oligonucleotide OD1 showing that only 66% of the cells express this receptor and that the fluorescence intensity is very low (few receptors on each cell). The third line shows the same results after 12 hours of incubation with the antisense oligonucleotides OD2 (52%) and OD3 (58%).

Additional experiments were performed to assess whether antisense oligonucleotides (OD3, OD2 and OD1 inhibited IL-4 receptor expression on RAJI cells by immunoprecipitation and Western blotting. It is to be noted in Fig. 9 that thirty million RAJI cells were cultured for 12 hours as previously described in complete medium with either 20µM of the sense oligonucleotide OD4 (first lane from the left), 10µM of

OD4 (second lane from the left), 10μM of the antisense oligonucleotide OD3 (third lane from the left), 10μM of the antisense oligonucleotide OD2 (fourth lane from the left), 10μM of the antisense oligonucleotide OD1 (fifth lane from the left), medium alone (sixth lane from the left and last lane on the right of the second gel), 20μM of the antisense oligonucleotide OD2 (first lane from the left of the gel on the right). The total protein was extracted and incubated with 2 μg of IL-4 overnight. Ten (10) μg/ml of anti-IL-4 antibody (R and D systems) coupled to 50 μl of protein A and Protein G-Sepharose™ was then added for two (2) hours at 20°C. The Sepharose™ beads were washed ten times and an agarose gel was used to separate remaining proteins. The remaining proteins were then transferred onto an Immobilon-P-millipore™ membrane and the Western revealed by a rabbit polyclonal anti-IL-4-R-alpha antibody (Santa Cruz biotechnology, Inc., cat# sc-684). The results show that sense oligonucleotides do not affect IL-4 receptor expression, that 10μM of the effective antisense oligonucleotides of the present invention inhibit IL-4 receptor expression and that 20μM of the antisense oligonucleotide OD2 is almost completely effective.

Dose response experiments were performed with the antisense oligonucleotide OD2 to determine the optimal concentration that block IL-4/IL-13 receptor expression in RAJI cells. It is to be noted in Fig. 10 which shows immunostaining experiments that OD2 also inhibited receptor expression when assessed by immunostaining studies. RAJI cells were cultured for 12 hours in complete medium containing 5μM OD2 (upper left), 10μM OD2 (middle left), 20 μM OD2 (lower left), no oligonucleotide (upper right), 10μM of the sense oligonucleotide for the same sequence as OD2 (middle

right) or 20 μM of the sense oligonucleotide for the same sequence as OD2 (lower right). Slides were fixed in methanol-acetone at -20°C for 10 min. After treatment with Tris-buffered saline containing universal blocking solution (DAKO) for 15 min., slides were incubated with an anti-IL-4 receptor serum (Santa Cruz biotechnology, Inc., cat# sc-684) at a final dilution of 1/200 overnight at 4°C., followed by incubation with 5 μg/ml alkaline phosphatase-labeled goat anti-rabbit IgG. Nuclei of cells were stained for 1 min. in Haematoxylin. Under these experimental conditions 20μM of OD2 almost completely inhibited IL-4 receptor expression.

As can be shown from Figs. 8 to 10, the antisense oligonucleotides of the present invention directed against the common subunit of the IL-4/IL-13 receptor are effective at inhibiting IL-4 receptor expression and it's functional component.

EXAMPLE III
Antisense oligonucleotides inhibiting the common beta subunit of IL-3, IL-5 and GM-CSF receptors

Interleukin-3, 5 and GM-CSF are important cytokines that are involved in eosinophil proliferation and survival. These cytokines are increased in asthma and atopic diseases and are also involved in the indefinite proliferation of certain neoplastic diseases. Interestingly, IL-3, IL-5 and GM-CSF share a common beta subunit that is involved in signal transduction.

Experiments were performed to assess whether antisense oligonucleotides of the present invention, directed against the common beta subunit of the IL-3, IL-5 and GM-CSF receptor, could inhibit the expression and the function of this receptor. TF-1 and U937 cells express high levels of GM-CSF receptors. In addition,

TF-1 cells are dependent on GM-CSF for survival. These cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin and l-glutamine at 37°C in 5% CO_2 (the TF-1 cells were supplemented with GM-CSF). For 12 hours they were either cultured in medium alone or medium with sense (107S: 5'-ACCATCCCGC TGCAGACCC-3' (SEQ ID NO:4)) or antisense (107A: 5'-GGGTCTGCAG CGGGATGGT-3'(SEQ ID NO:5)) oligonucleotides to the common beta subunit of the IL-3, IL-5 and GM-CSF receptor. The cells were retrieved and washed 3 times. RNA was then retrieved and the presence of the beta chain of the receptor was assessed by semi-quantitative RT-PCR. It is to be noted in Fig. 11 that the antisense oligonucleotides inhibit the expression of mRNA for the common beta receptor in TF1 cells (11A) and U937 cells (11B). In Fig. 11A, read from right to left, mRNA expression for Beta actin is shown in control, sense, and antisense treated cells (lanes 2,3,4); mRNA expression for the common receptor is shown in control, sense and antisense treated cells (lanes 5,6,7). The absence of a band in lane 7 is indicative of the effectiveness of the antisense oligonucleotide at inhibiting mRNA expression of the common Beta subunit in TF1 cells. In Fig. 11B, read from the right to left, mRNA expression for the common beta subunit is shown in control, sense, and antisense treated cells (lanes 2,3,4); mRNA expression for Beta actin is shown in control, sense and antisense treated cells (lanes 5,6,7). The absence of a band in lane 4 is indicative of the effectiveness of the antisense oligonucleotide at inhibiting mRNA expression of the common Beta subunit in U937 cells.

Additional experiments were performed to assess whether antisense oligonucleotides (107A) inhibited the

common beta subunit of IL-3, IL-5 and GM-CSF receptors in TF1 cells by immunoprecipitation and Western blotting. In Fig. 12, thirty million TF1 cells were cultured for 12 hours as previously described in complete medium with either 10 μM of the sense oligonucleotide 107S or the antisense oligonucleotide 107A (first lane from the left). The protein was extracted by immunoprecipitation with a monoclonal antibody against the GM-CSF beta chain receptor. The extracts were then transferred onto an Immobilon-P-millipore membrane after electrophoresis on a polyacrylamide gel, and the GM-CSF beta chain of the receptor was then revealed by a rabbit polyclonal anti-GM-CSF-R-Beta antibody. The results show that, at the same concentration (10μM), sense oligonucleotides do not affect the common beta chain expression, while the antisense oligonucleotides of the present invention inhibit the common beta subunit of IL-3, IL-5 and GM-CSF receptors.

Dose response experiments were performed with the antisense oligonucleotide 107A to determine the optimal concentration that would block TF1 cell growth. As seen in Fig. 13, antisense oligonucleotides of the present invention can be used to inhibit cell growth. TF1 cells were cultured in the presence of increasing concentrations of the oligonucleotides in serum free medium and then fetal bovine serum and GM-CSF were added to a final concentration of 10% and 1 ng/ml, respectively. The culture was performed for an additional 2 days and then cells were assayed for their capacity to reduce MTT dye over a four (4) hour period to a colored formazan product as an index of cell survival and proliferation. The results are expressed as a percentage of absorbance of MTT-derived formazan developed by untreated cells. Dot = mean ± SDEV. The

experiment was performed in triplicate. Absorbance was read at 570-595 nm.

It is to be noted in Fig. 14 that the antisense oligonucleotide 107A can significantly inhibit cell growth when compared to the sense probe or a control without antisense oligonucleotides. TF1 cells were cultured in the presence of the antisense oligonucleotide (first from right), the sense oligonucleotide (2nd from right), control medium (including GM-CSF, 3rd right) or medium without GM-CSF 4th from right) for 2 days and then cells were assayed for their capacity to reduce MTT dye as described above.

Other antisense oligonucleotides in accordance with the present invention have shown effectiveness at a concentration of 0.2 µMol. These antisense oligonucleotides are for example, but without limitation, the oligonucleotides 106: 5'-ggtctgcagc gggatggtt-3' (SEQ ID NO:6); 108: 5'-agggtctgca gcgggatgg-3' (SEQ ID NO:7); 110: 5'-gcagggtctg cagcgggat-3' (SEQ ID NO:8); 101: 5'-gcagcgggat ggtttcttc-3' (SEQ ID NO:9); 100: 5'-cagcgggatg gtttcttct-3'(SEQ ID NO:10); and 105: 5'-gtctgcagcg ggatggttt-3' (SEQ ID NO:11).

As can be shown from Figs. 12 to 14, the antisense oligonucleotides of the present invention directed against the common beta subunit of the IL-3, IL-5 and GM-CSF receptors are effective at inhibiting receptor expression and cell growth.

EXAMPLE IV
Antisense oligonucleotides inhibiting the CCR3 receptor for chemokines

There are two (2) considerations with regards to the CCR3 receptor: 1) it is expressed on the Th2 and not on the Th1 lymphocytes, 2) the CCR3 receptor is important for the recruitment of eosinophils into the

sites of allergic or asthmatic inflammation. The chemokines Eotaxin, MCP-4 and RANTES mediate most of their effects through the CCR3 receptor. These chemokines are present and increased in the lungs of patients with allergy and asthma (Lamkhioued et al., *Journal of Immunology*, 159:4593-4601, 1997). Fig. 15 shows that eotaxin is increased in epithelial cells and inflammatory cells in the lungs of patients with allergy and asthma. The expression and cellular distribution of eotaxin mRNA (15A to 15D) and protein (15E to 15H) in airways (15A, 15B, 15E) and BAL cells (15C, 15D, 15F to 15H) of asthmatic patients and normal controls have been assessed. Eotaxin mRNA expression is increased in asthmatic (15A) compared with normal (15B) airways. Prominent staining is observed in epithelial cells (Ep) and in many inflammatory cells (arrowheads) of the allergic asthmatic airway. Figs. 15C and 15D are representative examples of *in situ* hybridization of cytospin preparations of BAL cells obtained from an asthmatic patient and a normal control, respectively. Biopsy cell samples and biopsy sections were hybridized with an FITC-labeled antisense riboprobe complementary to eotaxin mRNA. The majority of positively hybridized cells in the BAL exhibited a morphology consistent with the macrophages (arrowheads). Fig. 15E shows immunohistochemical detection of eotaxin in a representative biopsy section of an asthmatic patient. Eotaxin immunoreactivity was visualized with the fast red chromogen and localized to the epithelial and inflammatory cells (arrowheads). Fig. 15F is a colocalization of eotaxin immunoreactivity (red) to CD-68 positive macrophages (brown) in BAL cells from an asthmatic patient by double immunohistochemistry. Examples of double positive cells are indicated with arrowheads. Figs.

15G and 15H show eotaxin immunofluorescent staining of BAL cells obtained from an asthmatic patient and a normal control, respectively. Note the eotaxin immunostaining in eosinophils (arrowheads).

Figs. 16A to 16H show that MCP-4 is also increased in epithelial and inflammatory cells in the lungs of patients with allergy and asthma. Results for asthmatic patients are reported in Figs. 16B, 16D, 16F, 16H, whereas Figs. 16A, 16C, 16E, 16G illustrates results obtained from normal control. MCP-4 mRNA and protein are increased in allergic asthma and found in epithelial and inflammatory cells. The tissue distribution of MCP-4 (mRNA and protein) expression in asthmatics and normal controls is shown. Positive *in situ* hybridization signals for MCP-4 mRNA were observed only when the antisense probes were employed. MCP-4 mRNA probe hybridized within the human bronchial epithelial cells in all sections (asthmatics and controls) that were examined. In asthmatics there is a strong hybridization signal for MCP-4 in epithelial cells and inflammatory cells (Fig. 16B). In contrast, in normal controls, a weak signal is observed in epithelial cells only (Fig. 16A). The MCP-4 protein was also detected in epithelial cells and the submucosa of biopsies of asthmatic airways (Fig. 16D). In normal airways, the epithelial cells and a few infiltrating cells were also positive for MCP-4 (Fig. 16C). No immunoreactivity was found in any cell type, when the first antibody was omitted or pre-absorbed with excess of recombinant MCP-4. The pattern of staining for MCP-4 protein appeared to be intracellular rather than membrane-bound, implying that these cells were synthesizing MCP-4. BAL cells from allergic asthmatic patients had a significantly increased number of cells expressing positive signals for MCP-4 mRNA (Fig. 16F).

Lymphocytes, macrophages and eosinophils stained positively in asthmatic subjects (Fig. 16F) whereas in normal controls, mRNA for MCP-4 was expressed only sporadically by macrophages and occasionally by epithelial cells (Fig. 16E). Results obtained with *in situ* hybridization were also confirmed by immunostaining (Figs. 16G and 16H) as the number of cells expressing MCP-4 was significantly increased in BAL from subjects with asthma (Fig. 16H) when compared to controls (Fig. 16G).

The contribution of the different chemokines present in the lungs of allergic patients with asthma to chemotaxis of purified eosinophils has also been assessed. Accordingly, lung bronchoalveolar lavage was performed in asthmatics. The supernatant was concentrated 10-fold with centricon™ columns. The inhibitory effect of antibodies directed against different chemokines on eosinophil migration in response to BAL fluid is assessed in Table 1. BAL fluid was preincubated with buffer, control Abs, polyclonal rabbit anti-eotaxin, anti-MCP-4, anti-RANTES Abs or a combination of these Abs for one hour before the chemotaxis assay was performed. The concentration of the eotaxin used in the BAL in each assay is indicated. Experiments were performed with a 48-well micro-chemotaxis chamber (NeuroProbe). Migration of human eosinophils was performed on a polycarbonate filter (5 μm pore size). Eosinophils (2 X 10^6 cells/ml) were resuspended in RPMI medium, loaded into the chambers, incubated at 37°C., 5% CO_2 for 60 min. and the filters were fixed and stained with a RAL kit (Labonord, France). Eosinophils were counted by microscopy in five selected high power fields (magnification x 400). For comparison of results from different chemotaxis assay, a chemotactic index (CI)

was calculated as follows: CI = (Counts-test sample)/(Counts-control medium). In the formula counts-test sample represents the number of migrated cells toward BAL or eotaxin, counts-control is the mean migration of cells in response to RPMI. The percentage of inhibition of locomotion and the confidence interval are presented for experiments performed on eosinophils obtained from 3 individuals. Percentage of inhibition was calculated by the formula: 100 - {(mean no. of migrated cells in Ab-treated fluids)/(mean number of migrated cells in untreated fluid)} x 100.

Table 1

Inhibitory effect of antibodies (Abs) on eosinophil migration in response to bronchoalveolar lavage (BAL) fluid

	% Inhibition of Migration							
BAL Eotaxin Concentration (pg/ml)	BAL/4	Anti-Eotax[a]	Anti-RANTES[a]	Anti-MCP4[a]	Anti-Eotax + Anti-RANTES[b]	Anti-Eotax + Anti-MCP4[b]	Anti-Eotax + Anti-RANTES + Anti-MCP4[b]	NRS
714.4	0	21.40	15.23	13.33	25.70	30.48	37.62	0.95
504.6	0	29.03	10.98	12.26	36.13	34.19	57.42	4.5
453.2	0	32.90	18.30	15.24	43.90	35.97	53.66	4.9

[a] $p < 0.05$ for the confidence interval compared with BAL sample alone; and
[b] $p < 0.01$ for the confidence interval compared with BAL sample alone.
NRS: Normal Rabbit Serum

In Table 1, the 3 chemokines that act mostly through the CCR3 receptor account for approximately 50% of the chemotaxis of eosinophils in asthmatic BAL.

These results show that chemokines (that act through the CCR3 receptor) are increased and important in allergic asthma and inhibition of the CCR3 receptor with antisense oligonucleotides is thus important in the therapy of allergy and asthma.

Furthermore, priming with the cytokine IL-5 (which acts through the IL-5 receptor) can either increase the chemotaxis of cells or the release of chemokines when the cells are stimulated.

Fig. 17 shows that priming of eosinophils with IL-5 increases the chemotaxis of eosinophils when they are stimulated with eotaxin. Preincubation of eosinophils with IL-5 (which acts through the IL-5 receptor) increases the chemotaxis induced by eotaxin at every dose tested. The peak of chemotaxis is higher with priming which suggests a synergistic effect of IL-5 on the effects of eotaxins. Dose-response curves show the chemotactic activity of purified human eosinophils to eotaxin (filled squares) and transmigration through a polycarbonate filter after preincubation with IL-5 (closed circles). Mononuclear cells and granulocytes were purified from peripheral blood by Ficoll-Paque (Pharmacia) density centrifugation. Granulocytes were obtained by dextran sedimentation. Human eosinophils were further purified by negative selection with anti-CD16 and anti-CD3-coated immunomagnetic microbeads using a Magnetic Cell Sorting System (Miltenyi Biotec) at 4°C. The degree of purity of eosinophil populations, estimated after staining with Giemsa, was between 92 and 100%. Results are presented as mean ± SD of 5 high power fields. Control serum had no effect on chemotaxis. Results

identified by "*" represent a probability of being different of $p < 0.01$ compared with unprimed eosinophils at each concentration of eotaxin.

Figs. 18a to 18I show that priming of eosinophils with IL-5 increases the amount of chemokines in the cells and increases their release after stimulation with immunoglobulin. Preincubation of eosinophils with IL-5 overnight increased the expression of eotaxin (Fig. 18A) and MCP-4 (Fig. 18B) when compared to controls (Fig. 18F). When eosinophils are stimulated with IgE-anti-IgE they will also release eotaxin (Fig. 18C, 18D, 18E) or MCP-4 (Fig. 18G, 18H, 18I). Eosinophils were purified as described above and incubated overnight with recombinant human IL-5 (1 ng/ml). This incubation increased eotaxin (Fig. 18A) and MCP-4 (Fig. 18B) in cells when compared to control cells incubated in medium alone (Fig. 18F). Stimulation of the eosinophils by a preincubation with IgE for 15 minutes then exposure to anti-IgE lead to a progressive release eotaxin (Figs. 18C, 18D, 18E) or MCP-4 (Figs. 18G, 18H, 18I) at 15 minutes (Figs. 18C, 18G), 2 hours (Figs. 18D, 18H) or 12 hours (Figs. 18E, 18I).

Accordingly, the combination of antisense oligonucleotides in accordance with the present invention, that are directed against different receptors (for example the IL-5 and the CCR3 receptors) have a synergistic effect in the therapy of allergy, asthma or neoplastic cell proliferation.

The antisense oligonucleotides of the present invention when compared to the use of soluble IL-4 receptors in allergy and asthma has the following advantages: a) as shown in example 1, the much smaller size of these molecules permits them to diffuse into the tissues and penetrate the cells that are expressing

the receptors (epithelial cells, smooth muscle cells); b) the use of an antisense oligonucleotide against the common sub-unit of the IL-4 and IL-13 receptor permits a broader effect by blocking the effects of IL-13 that are similar to those of IL-4 in many respects on IgE production, as IL-13 is also increased in allergy and asthma; and c) the combination of anti-receptor oligonucleotides against receptors for many cytokines (IL-3, IL-5 and GM-CSF or IL-4 and IL-13 or CCR3 (eotaxin, RANTES and MCP-4)) will permit broader effects in a disease where a certain individual's heterogeneity in the inflammatory cascade exist.

Furthermore, the antisense oligonucleotides of the present invention have the following advantages: a) the antisense anti-receptor oligonucleotides will act directly on tissue or inflammatory cells that are present at the site of administration and not indirectly by potentially blocking the release of mediators (if directed against the cytokines themselves); b) the antisense anti-receptor oligonucleotides will not be affected by diffusion of cytokines that are produced and increased in the blood of patients with allergy and asthma; and c) one antisense anti-receptor oligonucleotide of the present invention blocks the effects of 2 or 3 mediators which have been shown to be increased in allergy or asthma, thus having a broader effect than one antisense oligonucleotide directed only against one mediator or receptor and therefore being an advantage.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and

including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An antisense oligonucleotide for treating and/or preventing asthma, allergy, hypereosinophilia, general inflammation or cancer, said oligonucleotide being directed against a nucleic acid sequence coding for a receptor selected from the group consisting of a CCR3 receptor, a common subunit of IL-4 and IL-13 receptors, and a common subunit of IL-3, IL-5 and GM-CSF receptors.

2. The oligonucleotide of claim 1, wherein the nucleic acid sequence coding for the receptor is a nucleic acid coding for the common subunit of the IL-4 and IL-13 receptors.

3. The oligonucleotide of claim 1, wherein the nucleic acid sequence coding for the receptor is a nucleic acid coding for the common beta subunit of the IL-3, IL-5 and GM-CSF receptors.

4. The oligonucleotides of claim 1, wherein said oligonucleotide has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11.

5. A pharmaceutical composition for treating and/or preventing asthma, allergy, hypereosinophilia, general inflammation or cancer, said composition comprising at least one antisense oligonucleotide as defined in claim 1, 2, 3 or 4, in association with a pharmaceutically acceptable carrier.

6. Use of an oligonucleotide as defined in claim 1, 2, 3 or 4 for treating and/or preventing asthma, allergy, hypereosinophilia, general inflammation or cancer.

7. Use of a pharmaceutical composition as defined in claim 5 for treating and/or preventing asthma, allergy, hypereosinophilia, general inflammation or cancer.

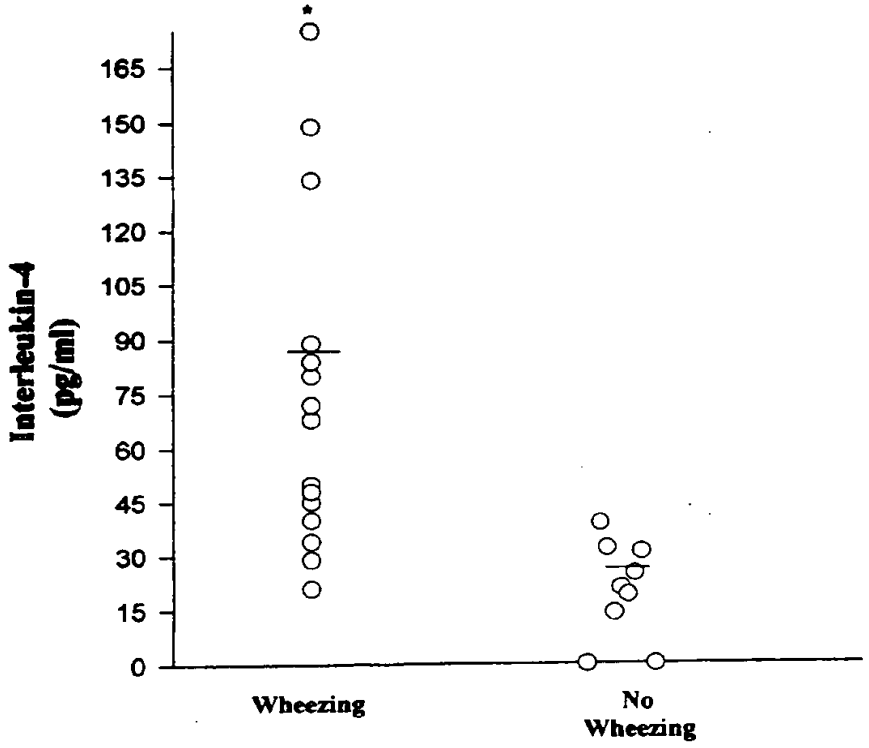

Interleukin-4
(pg/ml)
165
150
135
120
105
90
75
60
45
30
15
0
Wheezing
No
Wheezing

Fig. 1

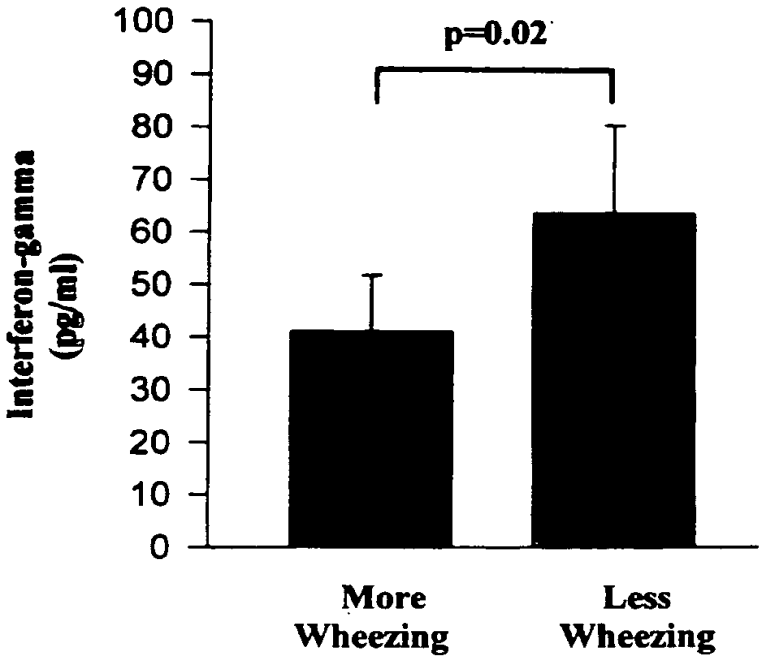

p=0.02
Interferon-gamma (pg/ml)
100
90
80
70
60
50
40
30
20
10
0
More Wheezing
Less Wheezing

Fig. 2A

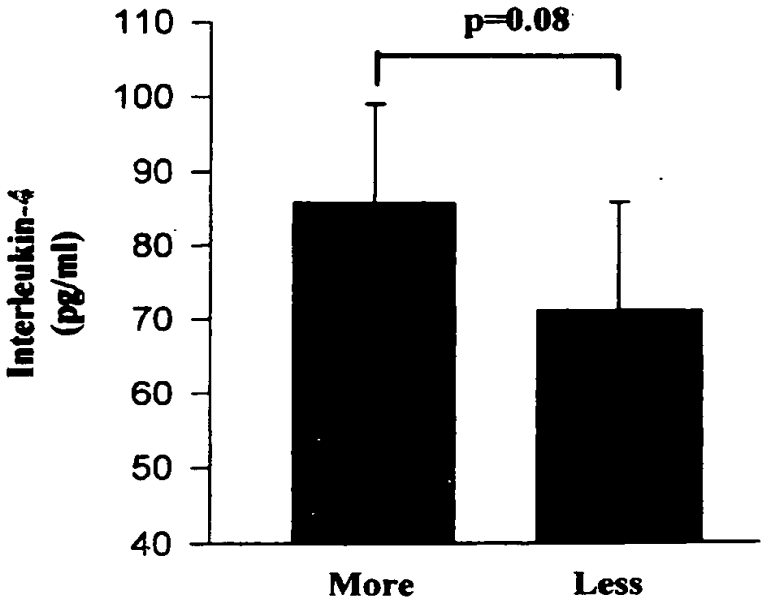

p=0.08
Interleukin-4 (pg/ml)
110
100
90
80
70
60
50
40
More
Less

Fig. 2B

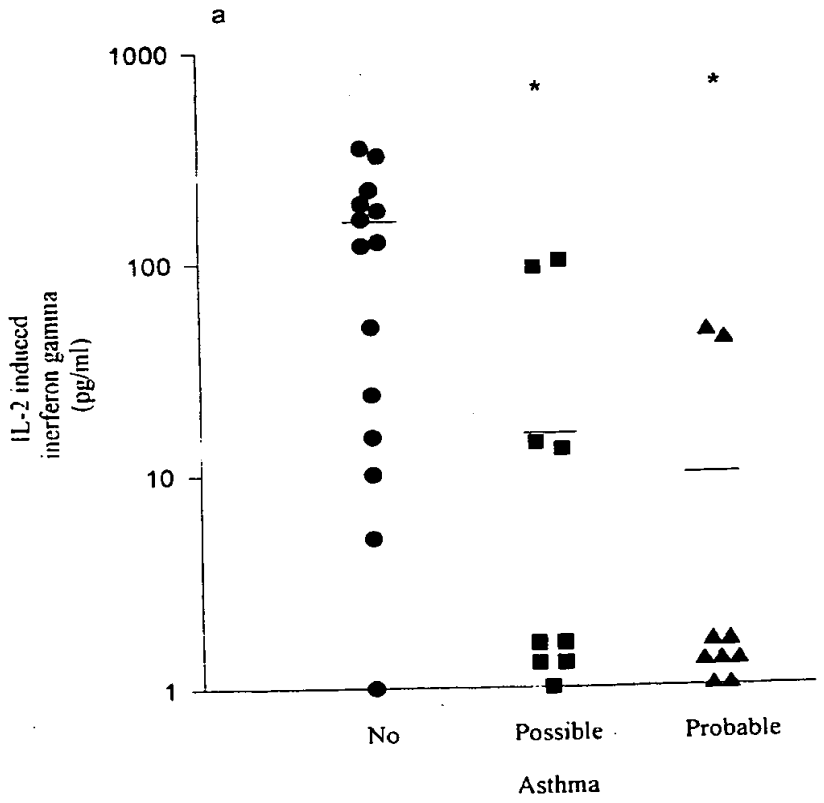

a
1000
100
10
1
IL-2 induced
interferon gamma
(pg/ml)
*
*
No
Possible
Probable
Asthma

Fig. 3A

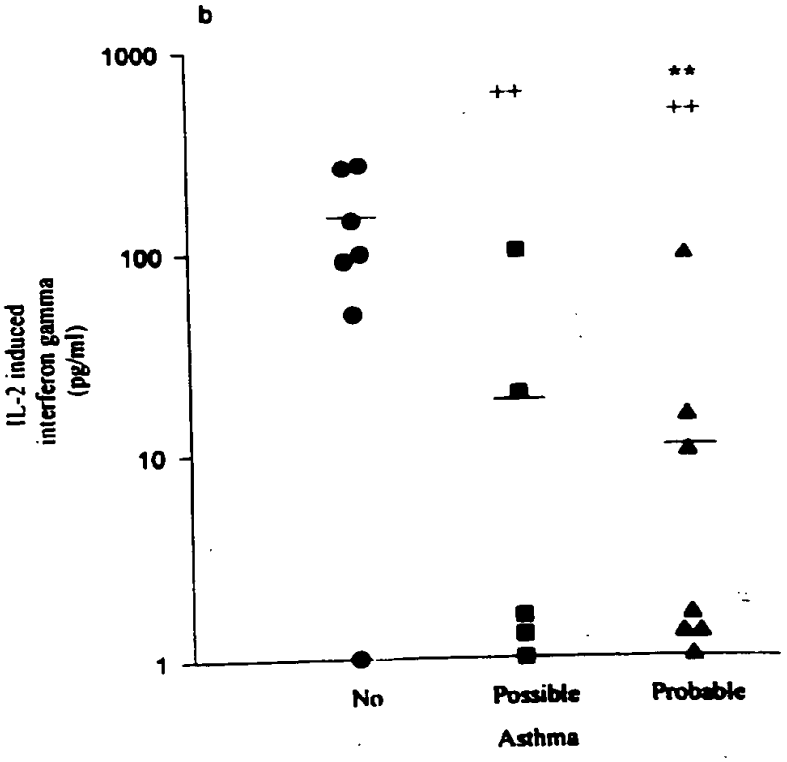

b
1000
100
10
1
IL-2 induced interferon gamma (pg/ml)
No
Possible
Probable
Asthma

Fig. 3B

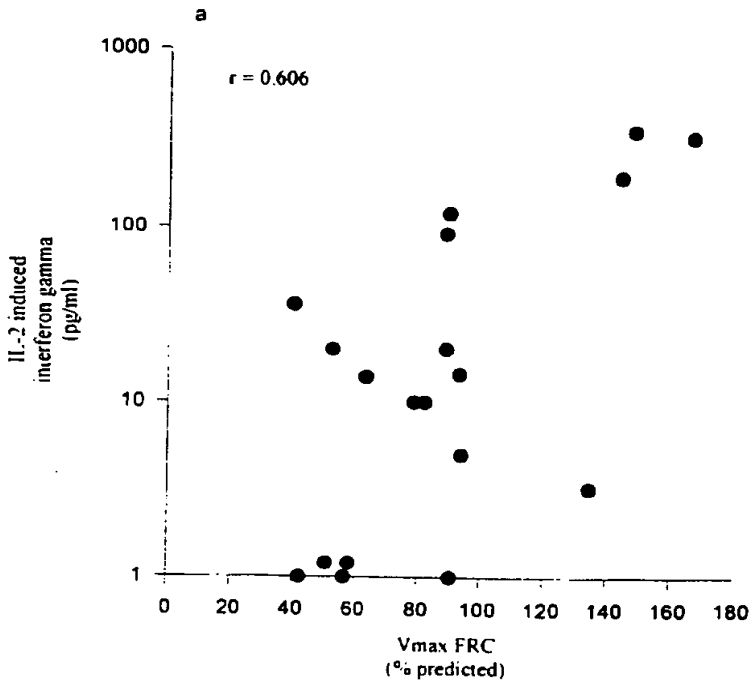

a
1000
100
10
1
r = 0.606
IL-2 induced
interferon gamma
(pg/ml)
0
20
40
60
80
100
120
140
160
180
Vmax FRC
(% predicted)

Fig. 4A

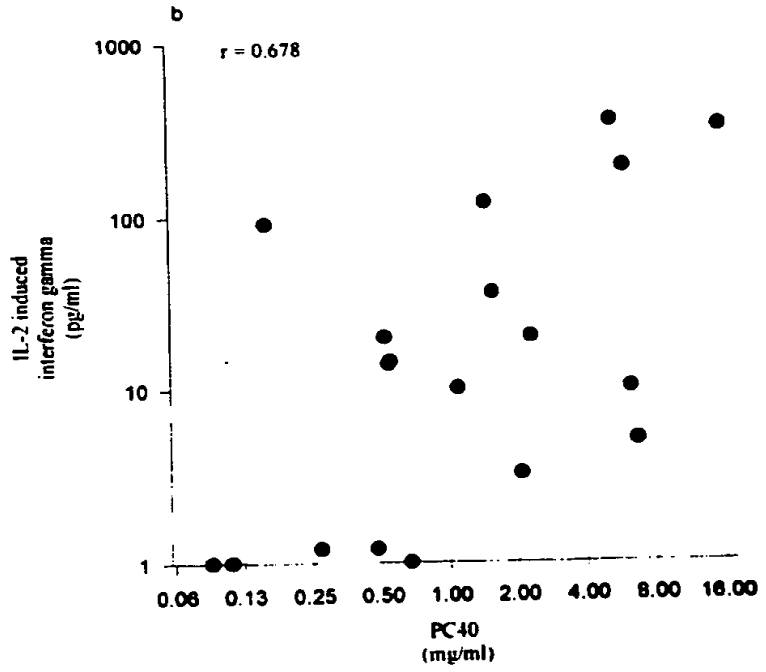

b
1000
r = 0.678
100
10
1
IL-2 induced interferon gamma (pg/ml)
0.06
0.13
0.25
0.50
1.00
2.00
4.00
8.00
16.00
PC40 (mg/ml)

Fig. 4B

Fig. 5A

Fig. 5B

Fig. 5C

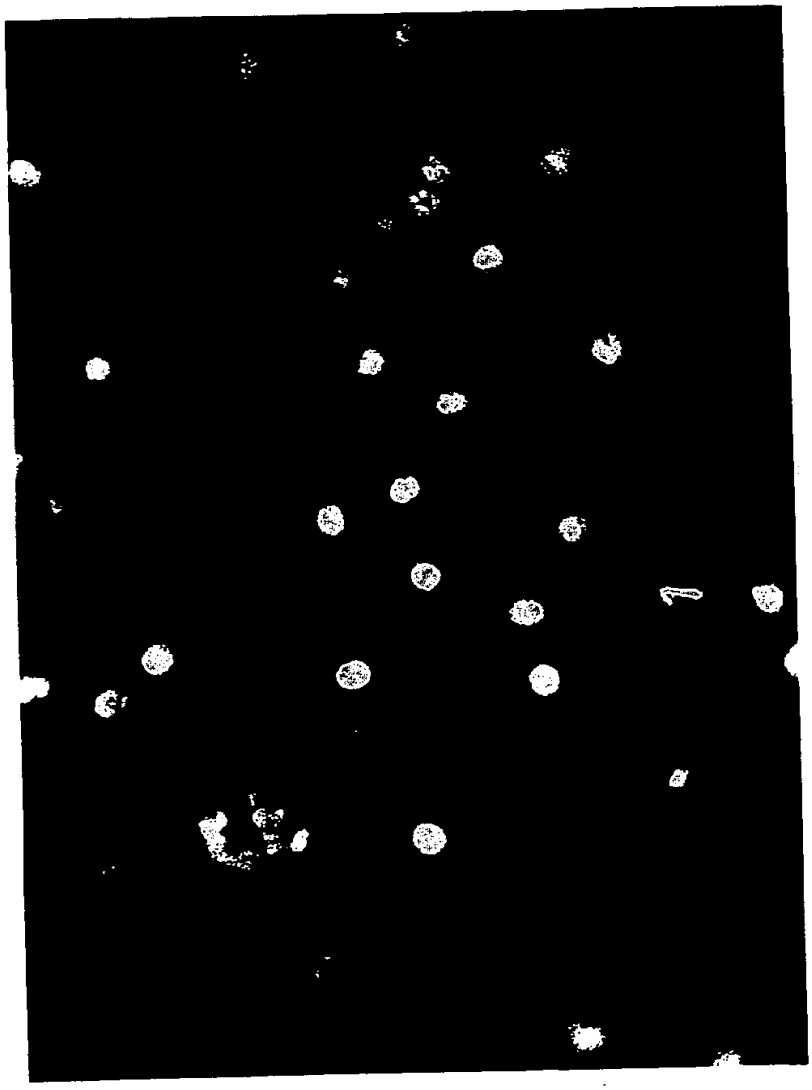


Fig. 6A

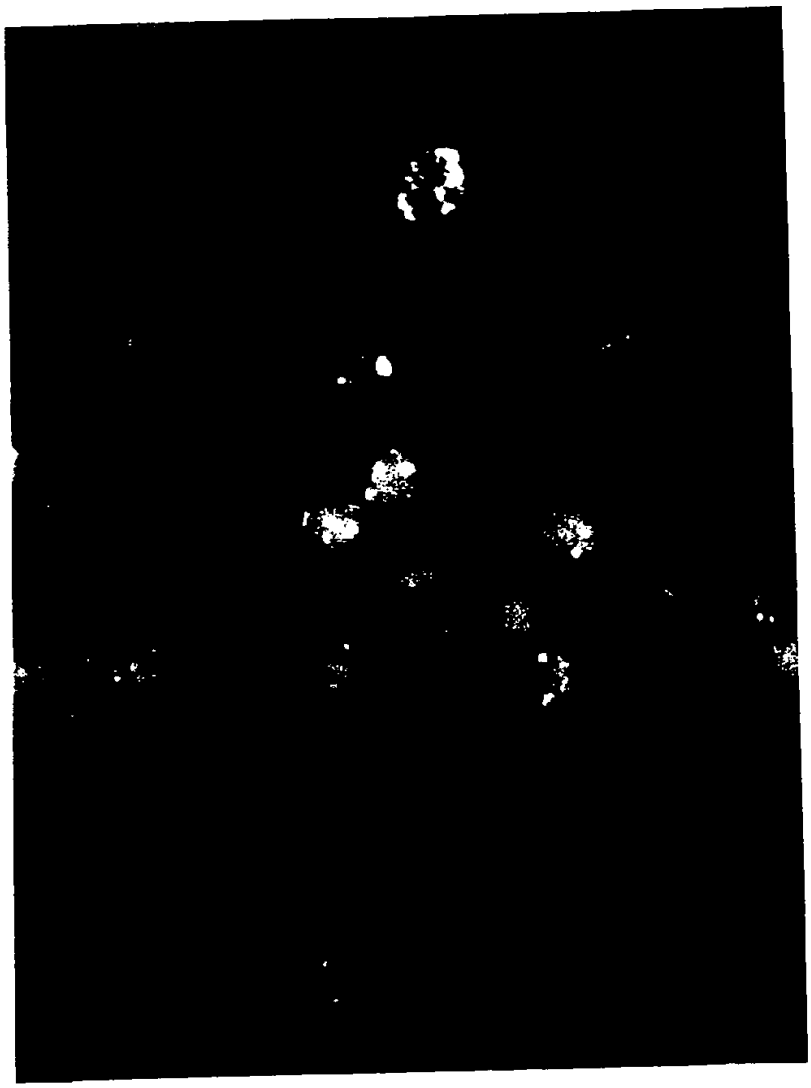


Fig. 6B

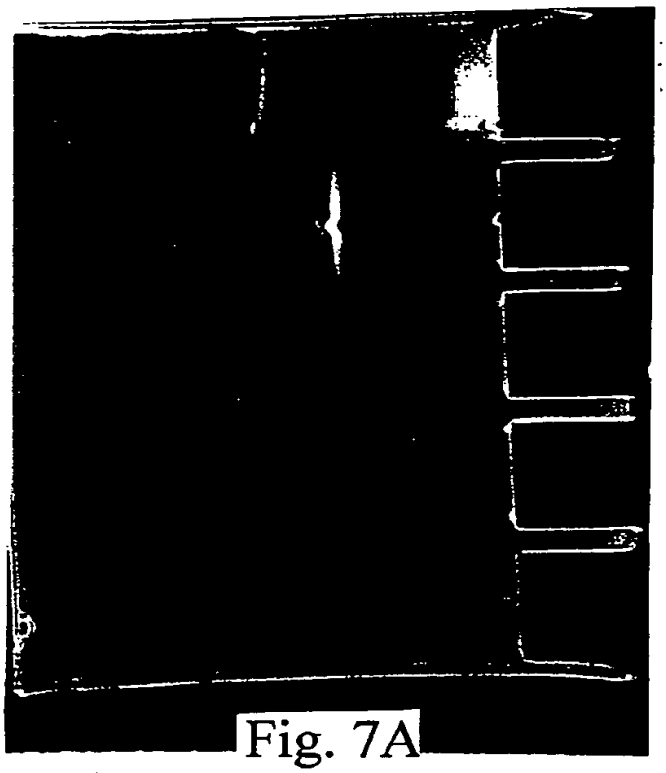


Fig. 7A

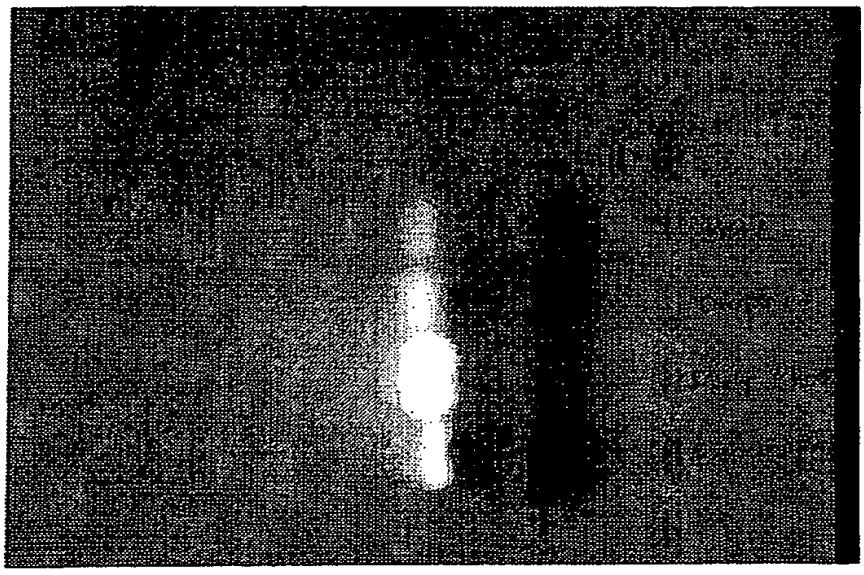


Fig. 7B

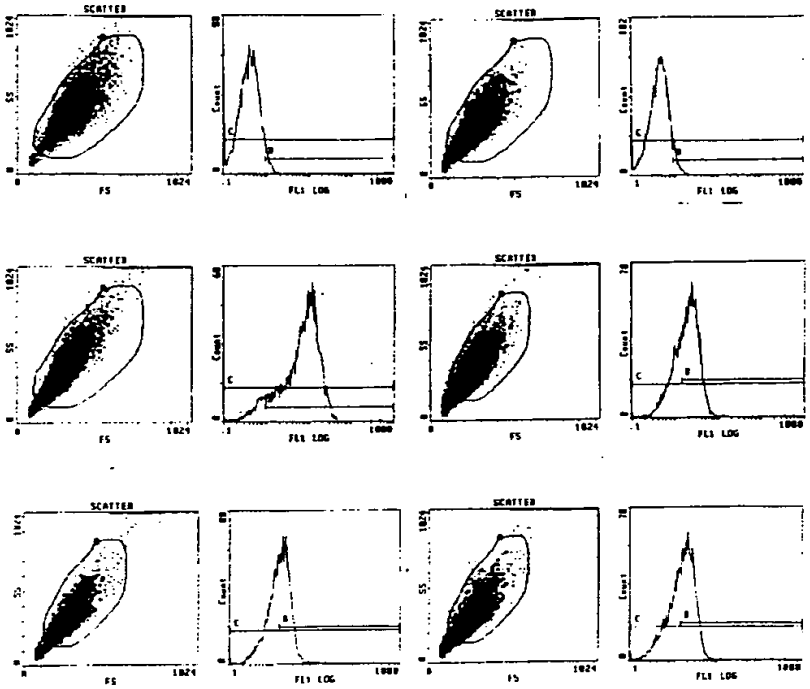


Fig. 8

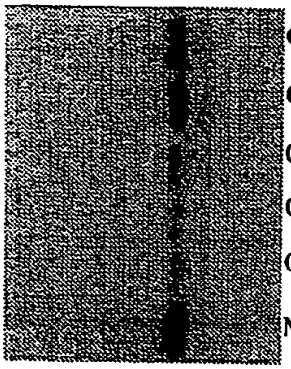

OD4s, 20 μM
OD4s, 10 μM
OD3as, 10 μM
OD2as, 10 μM
OD1as, 10 μM
Med
IL-4R

Fig. 9A

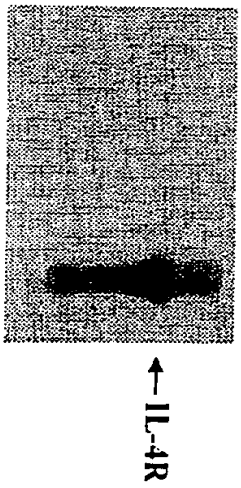

OD2as, 20 μM
Med
IL-4R

Fig. 9B

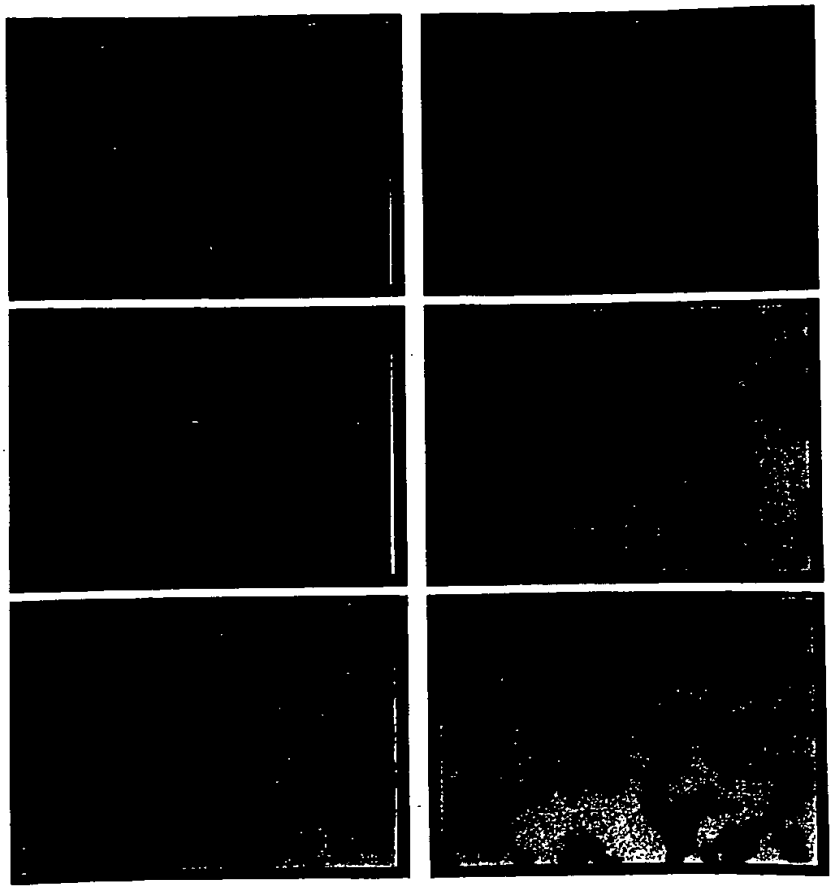


Fig. 10

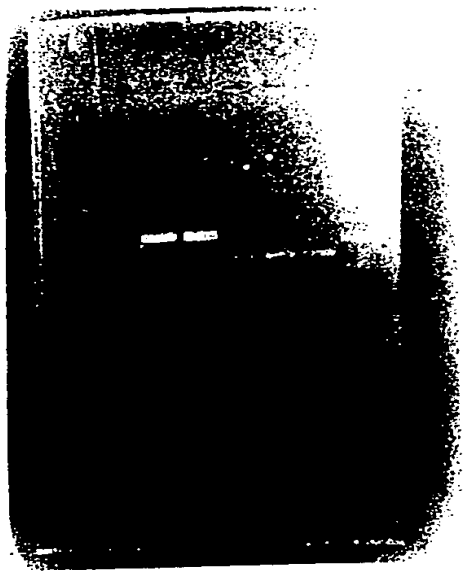
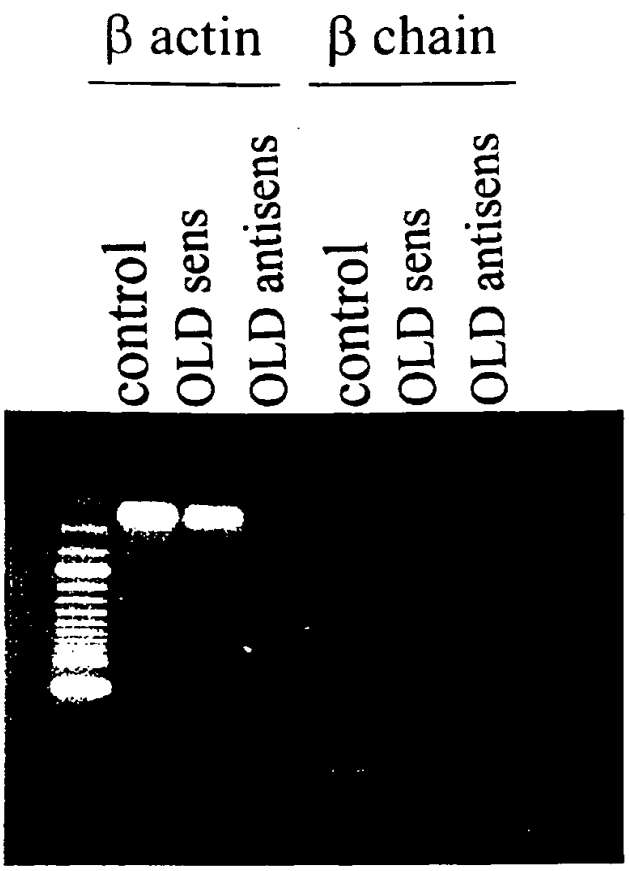


Fig. 11A


β actin
β chain
control
OLD sens
OLD antisens
control
OLD sens
OLD antisens

U 937

Fig. 11B

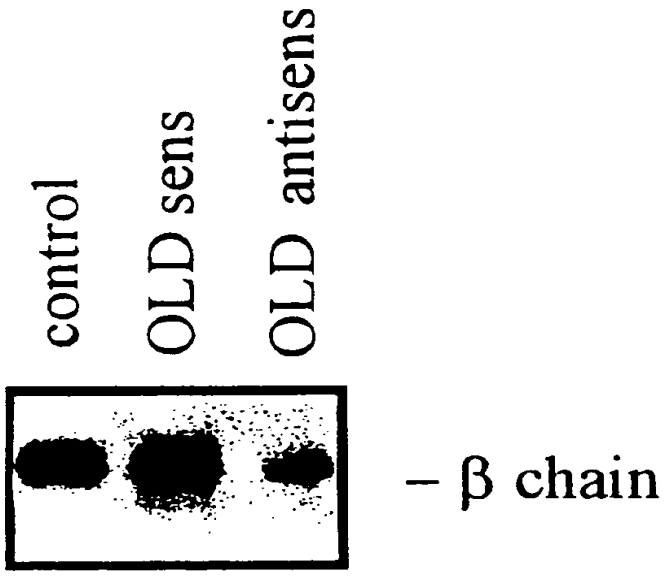


Fig. 12

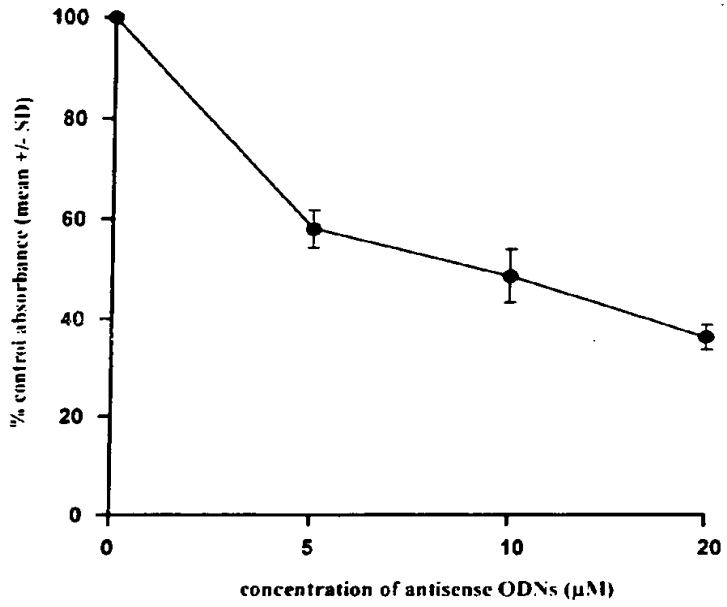

100
80
60
40
20
0
0
5
10
20
% control absorbance (mean +/- SD)
concentration of antisense ODNs (μM)

Fig. 13

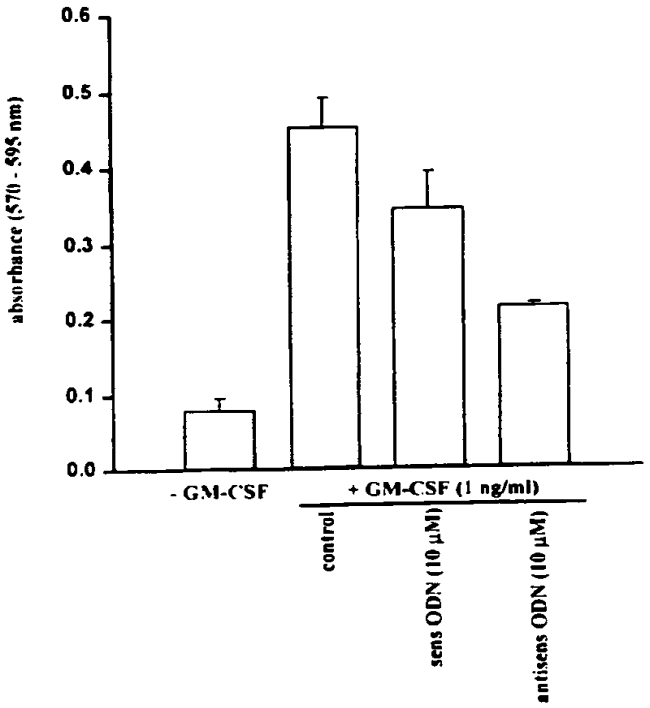

absorbance (570 - 595 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
- GM-CSF
+ GM-CSF (1 ng/ml)
control
sens ODN (10 µM)
antisens ODN (10 µM)

Fig. 14

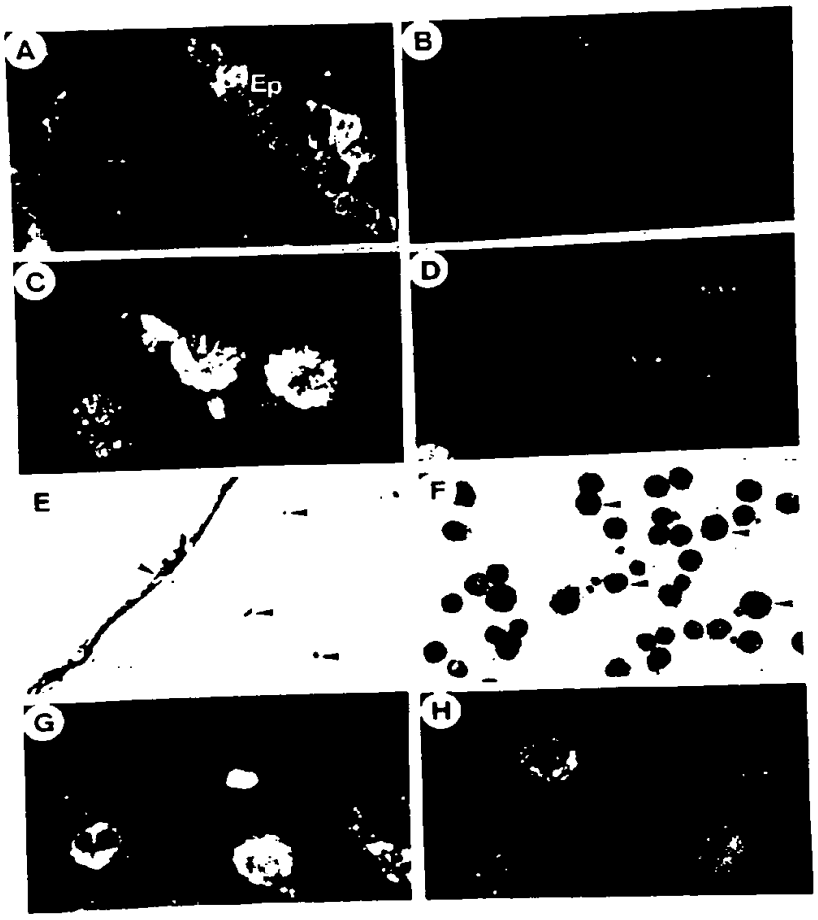


Fig. 15

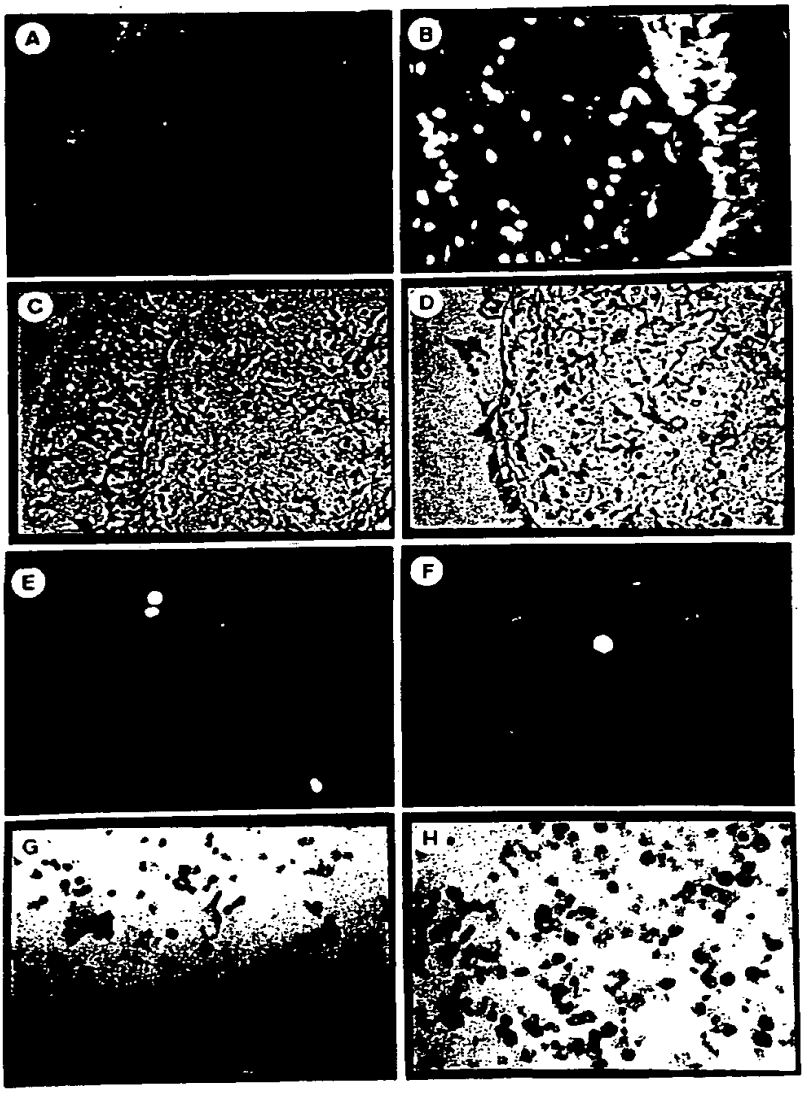


Fig. 16

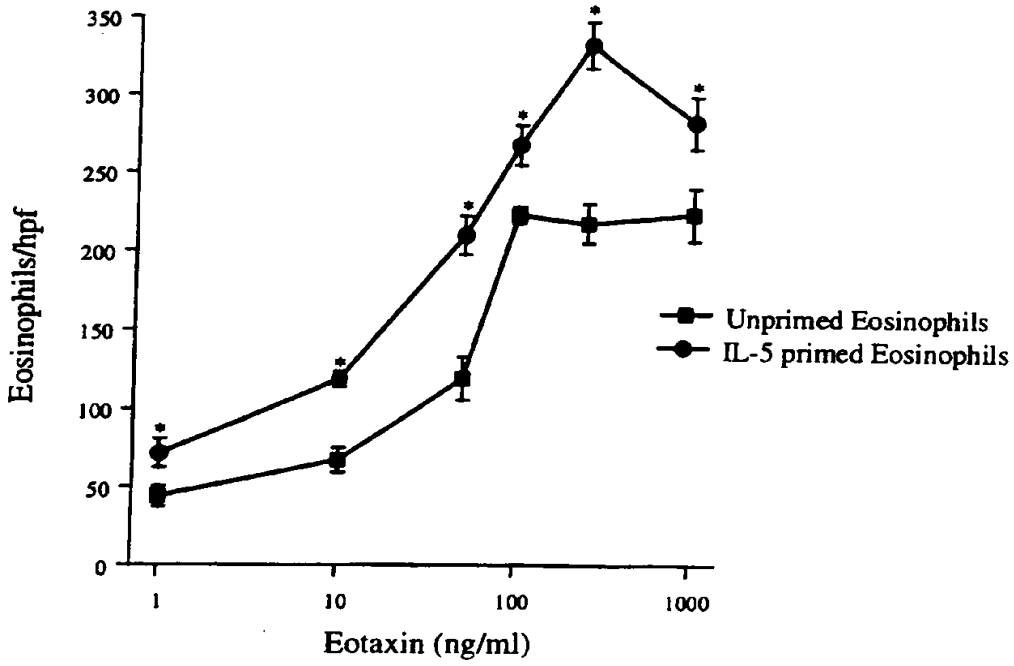

350
300
250
200
150
100
50
0
Eosinophils/hpf
1
10
100
1000
Eotaxin (ng/ml)
Unprimed Eosinophils
IL-5 primed Eosinophils

Fig. 17

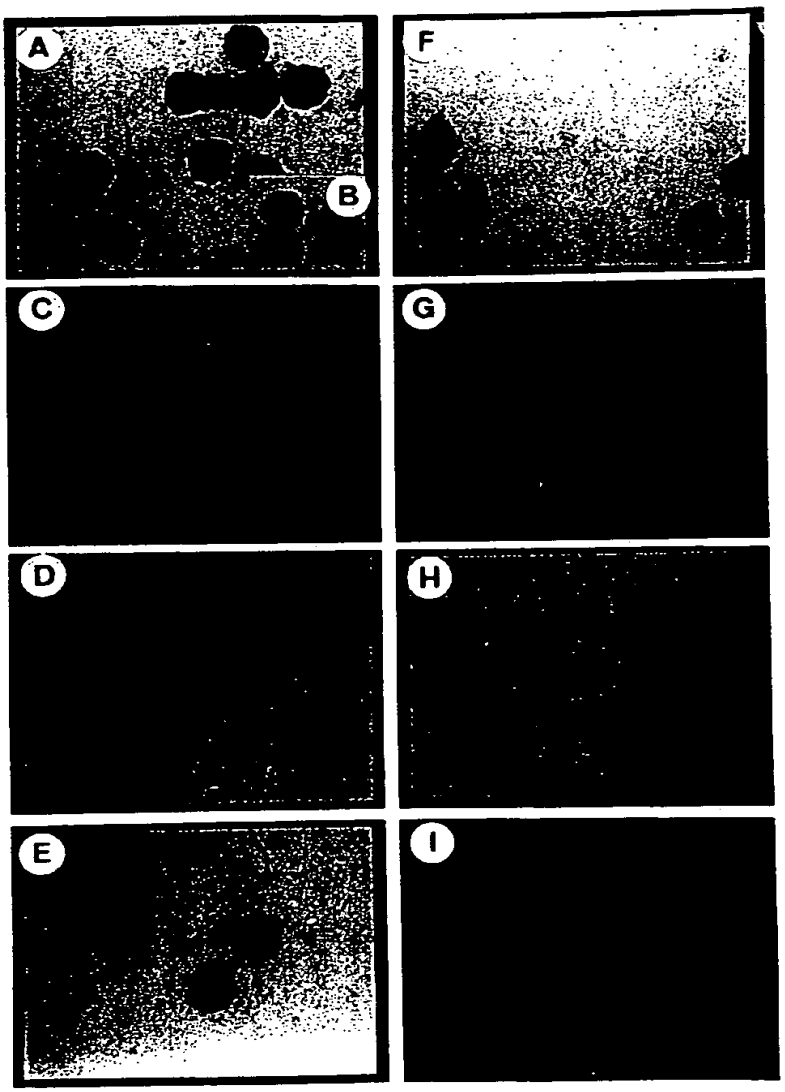


Fig. 18